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ANNOTATED BIBLIOGRAPHY OF SUBTILIN

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The genus Bacillus has a long record of observed antibiotic activity. This bibliography covers reports of the antibiotic activity produced by a particular strain of B. subtilis first studied from this aspect in the Western Regional Research Laboratory, and of the antibacterial agent, subtilin, concentrated from cultures of this organism. This strain of B. subtilis was originally obtained from N. R. Smith of the U. S. Department of Agriculture, Bureau of Plant Industry, Soils, and Agricultural Engineering, under the number 231. It is now deposited in the American Type Culture Collection as number 6633 and in the culture collection of the Northern Regional Research Laboratory as B-543.

Those articles originating from the Western Regional Research Laboratory are marked "WRRL" following the authors' names; those from the Medical School of the University of California, San Francisco 22, California, are marked "UC"; and those from the Department of Bacteriology, University at California at Los Angeles, California, are marked "UCLA".

Publications

Humfeld, H., and Feustel, I. C., WRRL. Utilization of asparagus juice in microbiological culture media. Proc. Soc. Expt. Biol. and Med. 54(2):232-235. 1943. B. subtilis cultures grown for 24 hours at 35° C. on asparagus butt juice medium were adjusted to pH 2.5 and sterilized for 10 minutes at 10 lbs. of steam pressure. These preparations possessed marked antibiotic activity against Staphylococcus aureus, Micrococcus conglomeratus, Lactobacillus casei, and the plant pathogen, Phytomonas michiganensis. Less activity was found against the Gram-negative plant pathogens Phytomonas juglandis and Erwinia amylovora. (Reprints exhausted.)

Jansen, E. F., and Hirschmann, D. J., WRRL. Subtilin--An antibacterial product of Bacillus subtilis. Culturing conditions and properties. Arch. Biochem. 4(3):297-309. 1944. B. subtilis was grown in surface cultures on media containing sucrose, mineral salts, and several organic nitrogen sources including asparagine, glutamic acid, aspartic acid, casein, and tryptone. The addition of manganese (1 p.p.m.) proved essential for high antibiotic activity. Wide variations were noted in qualitative serial dilution tests of antibacterial activity against Staphylococcus aureus, Micrococcus conglomeratus, and Lactobacillus casei of cultures acidified to pH 2.5 and sterilized for 10 minutes at 10 lbs. of steam pressure. The material having antibacterial activity was named subtilin, but was believed to consist of more than one substance. A crude concentrate was prepared by centrifuging and vacuum-drying cultures adjusted to pH 4.7. The active material was reported to be soluble in aqueous ethanol but not in 95 percent ethanol, to be diffusible, to be heat-labile under alkaline conditions but relatively stable at pH 2.5, and to be inactivated by light and by formaldehyde. (Reprints exhausted.)

Anonymous, WRRL. Production, concentration, properties, and assay of the antibiotic, subtilin. Mimeographed Circular AIC-106, Bureau of Agricultural and Industrial Chemistry, U. S. Department of Agriculture. 1946. A summary of the work on subtilin done at the Western Regional Research Laboratory prior to 1946. B. subtilis was grown on asparagus butt juice concentrate diluted to 8 percent solids. The initial pH of the medium was 6.5. The cultures were incubated for 28 hours at 37° C., at which time the pellicle (which contained most of the antibiotic activity) was separated from the culture liquor by straining. Subtilin was concentrated approximately 100-fold as compared to the original B. subtilis pellicle (dry basis) by extracting the moist pellicle with aqueous ethanol (final alcohol concentration 60-70 percent), concentrating in vacuo to remove the alcohol, salting out the active material, extracting the dried salt precipitate with 95 percent ethanol, and fractionating the residue with 25 percent acetic acid. The active acid-soluble material was dried by lyophilization to give a light brown powder characterized by being precipitated by low salt concentrations and by being appreciably soluble only in acid solution. It was insoluble in many organic solvents, including acetone, ether, petroleum ether, chloroform, and amyl acetate. It was most stable in acid and relatively unstable in alkaline solution. Alcoholic culture extracts and concentrates were assayed by a quantitative bacteriostatic method, which is described. Aliquots of unknowns and reference standard concentrate were made to 5 ml. volume in pH 2.5 HCl diluent in nonsterile culture tubes. 10-ml. aliquots of medium inoculated with Staphylococcus aureus were distributed in the culture tubes, which were incubated for 4 hours in a water bath at 37°. The cultures were stabilized by autoclaving, and turbidities were read photometrically to permit quantitative comparison of standards and unknowns. The assay medium was "Medium II" of Schmidt and Moyer (Jour. Bact. 47, 199-208, 1944); it was prepared in 150 percent strength to allow for dilution by the test solutions.

Lewis, J. C., Feeney, R. E., Garibaldi, J. A., Michener, H. D., Hirschmann, D.J., Traufler, D. H., Langlykke, A. F., Lightbody, H. D., Stubbs, J. J., and Humfeld, H., WRRL (authors Traufler and Langlykke, NRRL). Subtilin production in surface cultures. Arch. Biochem. 14(3):415-425. 1947. The highest yields of subtilin in shallow-layer cultures (as determined by the antibiotic activity of aqueous ethanol extracts) were obtained with a beet molasses medium, which required the addition of $(NH_4)_2HPO_4$ (0.8 percent) and manganese (50 p.p.m.). Good yields were obtained on media prepared from asparagus butt waste press juice, molasses and grain worts, and corn steep liquor. An incubator temperature of 35° C., with peak temperatures in the cultures 4° to 8° C. higher, was approximately optimal. Maximum yields were usually attained in cultures 1 to 2 cm. deep after 24 to 48 hours. Prolonged incubation of asparagus and beet molasses media resulted in a drop in antibiotic activity. The distribution of subtilin between pellicle and culture filtrate varied widely for different media. Two other strains of B. subtilis did not produce appreciable amounts of subtilin.

Stubbs, J. J., Feeney, R. E., Lewis, J. C., Feustel, I. C., Lightbody, H. D., and Garibaldi, J. A., WRRL. Subtilin production in submerged culture. Arch. Biochem. 14(3):427-435. 1947. Excellent yields of subtilin were produced in fermenters of one-liter capacity equipped with high-speed stirrers for dispersion of air. Asparagus-juice concentrate, when diluted to 8 percent solids, steamed for 30 minutes, adjusted to pH 7.0, inoculated, and incubated at 35° C. under aeration at a rate of one volume of air per minute for 10 hours, yielded activity equivalent (per liter of culture liquor) to 1000 to 1200 mg. of the reference standard lot (L1263) of partially purified subtilin. Subtilin was also produced in similar yield but at a slower rate on media prepared from beet molasses.

Lewis, J. C., Humphreys, E. M., Thompson, P. A., Dimick, K. P., Benedict, R. G., Langlykke, A. F., Lightbody, H. D., WRRL (authors Benedict and Langlykke, NRRL). The microbiological assay of subtilin. Arch. Biochem. 14(3):437-450. 1947. Subtilin was assayed by a turbidimetric method which depended on growth inhibition of Micrococcus conglomeratus, Streptococcus faecalis, or Staphylococcus aureus. The cultures were indubated for 4 or 5 hours at 37° C. under nonsterile conditions. The antibiotic activity of crude culture extracts of B. subtilis and of partially purified lots of subtilin could not be accounted for entirely in terms of a single active substance. Sixty to 70 percent ethanol was optimal for extraction of subtilin from crude cultures. The extraction was essentially complete in 15 minutes. Crude cultures lost substantial amounts of activity on steaming for 1 to 2 hours; the rate of destruction was faster at pH 6.4 to 7.1 than at 2.5.

Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., WRRL. Purification and some properities of subtilin. Arch. Biochem. 1947. (In press). Subtilin was obtained by extraction with 70 percent ethanol from pellicles produced by B. subtilis on asparagus butt juice medium. The extract was concentrated in vacuo to remove ethanol, whereupon the active material precipitated. To remove inactive contaminants the dried filter cake was extracted successively with 95 percent ethanol, and with 85 percent ethanol containing 1 percent each of acetic acid and NaCl. The active material was then obtained in solution by extracting the residue with 0.16 M acetate at pH 4.6. The extracts were treated with filtering aid (Hyflo Super-Cel)1/, deionized with exchange resins, concentrated, and lyophilized to yield a dull white powder. The subtilin preparation was soluble to more than 10 percent in salt-free acidified water, but to less than 0.5 percent at pH 6 to 9. In the presence of 0.5 percent NaCl at acid pH the solubility was less than 0.5 percent. The preparation was soluble in 0 to 80 percent ethanol, and in methanol, but not in dry ethanol, butanol, amyl alcohol, acetone, ether, petroleum ether, or chloroform. It was about 0.5 percent soluble in n-butanol saturated with water. It diffused fairly rapidly through Cellophane. The preparation contained 14 percent total (Kjeldahl) nitrogen. The amino nitrogen content of 1.6 percent increased to about 11 percent after acid hydrolysis. It contained 4.2 percent sulfur and a trace of phosphorus. It was levorotatory. Subtilin was inactivated by alkali, and the activity decreased on incubation with pepsin and trypsin. Dry subtilin preparations lost activity when stored for a period of months at room temperature, but dilute solutions at pH 2.5 appeared stable when stored in the refrigerator.

Dimick, K. P., Alderton, G., Lightbody, H. D., and Fevold, H. L., WRRL. A method for purification of subtilin (abstract). Fed. Amer. Socs. Expt. Biol. Proc. 6(1):247-248. 1947. An improved method is described in which surface or submerged cultures of B. subtilis, adjusted to pH 2.5 with HCl, were extracted with 1/2 to 1 volume of n-butanol. The two phases were separated in a Sharples1/ centrifuge and 1/2 volume of petroleum ether was added to the aqueous butanol extract. The butanol-petroleum ether mixture was extracted 3 times with 1/10 volume of 1 percent acetic acid. Sodium chloride was added to the combined aqueous extracts to 6 percent concentration. A precipitate separated on the surface and

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1/ The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

was skimmed off. It was washed with petroleum ether and the excess salt solution was removed by filtration. The filter cake was dried by lyophilization and extracted with 95 percent alcohol (5 or 6 times with 20 ml. per gram) and subsequently in a similar manner with 85 percent ethyl alcohol containing 1 percent NaCl and 1 percent acetic acid. The extracts were discarded. The alcohol-insoluble residue was dissolved to a concentration of 1 percent in distilled water. The pH was adjusted to 4.5 with dilute NaOH and 0.4 percent NaCl added. The precipitate was removed by centrifugation, redissolved, and reprecipitated as before. This was repeated three or four times. The active extracts were deionized by ion-exchange resins and dried by lyophilization, or the active material was precipitated with 10 percent NaCl and subsequently dissolved, deionized, and dried.

Feeney, R. E., Humphreys, E. M., Lightbody, H. D., and Garibaldi, J. A., WRRL. Nutritional studies on the formation of subtilin by Bacillus subtilis in surface cultures (abstract). Fed. Amer. Socs. Expt. Biol. Proc. 6(1):250. 1947. Moderate growth and antibiotic yields equivalent to 600 to 800 mg. per liter of the reference standard lot (L1263) of partially purified subtilin were obtained after 60 to 72 hours of incubation in shallow layer cultures of the following synthetic medium: sucrose, 10 percent; asparagine, 0.2 percent; glutamic acid, 0.2 percent; citric acid, 0.05 percent; Na_2SO_4, 0.4 percent; $(NH_4)_2HPO_4$, 0.8 percent; NaCl, 0.03 percent, and traces of other salts. Requirements for potassium, magnesium, manganese, iron, and zinc were demonstrated. Calcium exerted a markedly deleterious effect. A zinc-free medium was prepared (by extraction with diphenylthiocarbazone in carbon tetrachloride) which permitted only slight growth and subtilin yields of less than 20 mg. per liter. This medium gave a linearly increasing response in subtilin yields when 0.03 to 1 p.p.m. of zinc was added. Maximum yields were obtained over the range of 1 to 10 p.p.m. of added zinc.

Feeney, R. E., Lightbody, H. D., and Garibaldi, J. A., WRRL. Zinc as an essential element for growth and subtilin formation by Bacillus subtilis. Arch. Biochem. (In press). Zinc was found essential for growth and subtilin formation by B. subtilis in shallow layer stationary cultures. The minimum requirement for zinc was approximately 1.0 p.p.m. This appears to be the first demonstration of an absolute requirement for zinc in bacterial growth. Cadmium, the only element capable of substitution for zinc, was only partially effective.

Lewis, J. C., and Jansen, E. F., WRRL. Enhancement of subtilin activity by methylation (abstract). Fed. Amer. Socs. Expt. Biol. Proc. 6(1):270. 1947. The bacteriostatic activity of subtilin against Micrococcus conglomeratus, Staphylococcus aureus, and Streptococcus faecalis was increased approximately 4-, 2-, and 5-fold, respectively, by treatment of the subtilin with dilute HCl in methanol. The greatest enhancement was found after 6 to 20 hours of incubation at room temperature of a 1 percent solution in 0.6 N HCl-90 percent methanol or after 20 to 40 hours in a 0.03 N HCl-absolute methanol. The isolated product contained 2.0 percent $-OCH_3$ by Zeisel as compared with a negligible test (0.15 percent) in the unmodified subtilin. The activity decreased markedly on more prolonged or more drastic treatment. Treatment of subtilin with diazomethane resulted in inactivation.

Michener, H. D., and Snell, N., WRRL. Antifungal activity of Bacillus subtilis cultures (approved for presentation before the American Association for the Advancement of Science, Botanical Society of America, at San Diego, Calif., June, 1947. Cultures of subtilin-producing B. subtilis possessed antifungal activity, which was not exhibited by partially purified subtilin preparations. A single extraction with butanol at pH 2.5 removed most of the activity against Aspergillus niger but not against Rhizopus solani, thus indicating that at least two antifungal substances were present.

Salle, A. J., and Jann, G. J., UCLA. Subtilin--An antibiotic produced by Bacillus subtilis. I. Action on various organisms. Proc. Soc. Expt. Biol. and Med. 60, 60-64. 1945. Partially purified subtilin was found to inhibit growth of a number of Gram-positive bacteria, the Gram-negative Neisseria catarrhalis and N. gonorrhoeae, certain acid-fast bacteria including Mycobacterium tuberculosis, and certain pathogenic fungi. The effect on M. tuberculosis was bacteriostatic at low and bactericidal at higher concentrations.

Salle, A. J., and Jann, G. J., UCLA. Subtilin--Antibiotic produced by Bacillus subtilis. II. Toxicity of subtilin to living embryonic tissue. Proc. Soc. Expt. Biol. and Med. 61, 23-24. 1946. Partially purified subtilin possessed a low toxicity for embryonic chick heart tissue cultures. Exposure to a 0.2 percent solution for 10 minutes at 37° C. was required to kill the cultures. A concentration of 0.01 percent was required to kill Staphylococcus aureus under similar conditions.

Salle, A. J., and Jann, G. J., UCLA. Subtilin--Antibiotic produced by Bacillus subtilis. III. Effect on type III pneumococcus in mice. Proc. Soc. Expt. Biol. and Med. 62, 40-42. 1946. Partially purified subtilin in repeated doses of 0.05 mg. injected intraperitoneally quickly cured experimental type III pneumococcus infections induced in mice by intraperitoneal inoculation, without apparent toxic reaction in the animals. A group untreated until 9 hours after infection were in very bad condition before treatment, but appeared almost normal after 2 or 3 injections.

Salle, A. J., and Jann, G. J., UCLA. Subtilin--Antibiotic produced by Bacillus subtilis. IV. Effect of subtilin on the course of experimental anthrax infections in guinea pigs. Proc. Soc. Expt. Biol. and Med. 63, 41-42. 1946. Partially purified subtilin in doses of 6 mg. injected intraperitoneally at frequent intervals over a period of 11 days protected guinea pigs from experimental anthrax infections induced by intraperitoneal inoculation, without apparent toxic reaction in the animals. Initial treatments were made 3, 6, and 9 hours after inoculation. Untreated controls died in about 3 days.

Anderson, H. H., Villela, G. G., Hansen, E. L., and Reed, R. K., UC. Some physical and biologic properties of subtilin and other antibiotics. Science 103(2675):419-420. 1946. Partially purified subtilin proved active in vitro against Lactobacillus plantarum, Endamoeba histolytica and its associated bacterium 't', and Trypansoma equiperdum. The concentrations required for antibiotic effect also lowered surface tension appreciably. Intravenous injection of 1 percent solution in mice gave an LD_{50} of 60 ± 3 mg. per kg.; subcutaneous injection gave an LD_{50} of 670 ± 30 mg. per kg. When given intragastrically, 5.0 grams/kg. killed. One percent solution instilled into the rabbit's eye was nonirritating.

Anderson, H. H., and Wong, S. C., UC. Antibiotics in experimental tuberculosis. Tuberculology 8(3):77-82. 1946. Partially purified subtilin at 2.5 p.p.m. prevented growth of virulent strains of Mycobacterium tuberculosis in a modified Dubos medium. The partially purified preparations used gave a marked anaphylactic reaction with guinea pigs. Topical application of subtilin was ineffective in treating experimentally induced tuberculous infections of the cornea of rabbits. Daily subcutaneous injections of 6 mg. of subtilin continued over a period of 6 weeks failed to affect the course of experimentally induced tuberculosis in Syrian hamsters. Therapy was initiated 8 days after infection. One particular lot of subtilin was well-tolerated; two others gave marked local tissue toxicity. It was believed that failure of the therapy was related to precipitation by physiological concentrations of NaCl. Such concentrations reduced the in vitro antibiotic activity of subtilin on Lactobacillus plantarum.

Goodman, J. J., and Henry, A. W., Dept. of Plant Science, Univ. of Alberta, Edmonton, Canada. Action of subtilin in reducing infection by a seed-borne pathogen. Science 105, 320-321. 1947. Barley seeds exposed simultaneously to a 0.1 percent solution of partially purified subtilin and Xanthomonas translucens cerealis for 24 hours did not yield infected seedlings, but with 0.02 percent subtilin, infection approached that of controls without subtilin. Only partial protection was obtained when the seeds were infected, dried, and subsequently treated with subtilin.

Anonymous, WRRL:

Production of subtilin--A new antibiotic. Drug and Cosmetic Industry 60, 478-479. 1947.

Subtilin output and purification. Oil, Paint, and Drug Reporter 151, 5, 55.1947.

Subtilin from asparagus waste. Chem. and Engin. News 25, 1070-1071. 1947.

(These are announcements and brief abstracts of work done at the WRRL on production, purification, and assay of subtilin.